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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/02	A1	(11) International Publication Number: WO 93/04690 (43) International Publication Date: 18 March 1993 (18.03.93)
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(54) Title: METHOD FOR THE TREATMENT OF THE COMPLICATIONS AND PATHOLOGY OF DIABETES (57) Abstract <p>The present invention provides a method for the treatment of the complications and pathology of diabetes. The method involves the administration to a diabetic subject of a composition comprising a compound selected from the group consisting of (β-Ala-His)_n, (Lys-His)_n, a compound of the formula R₁-X-R₂, pharmaceutically acceptable salts thereof and combinations thereof; and a pharmaceutically acceptable carrier, in which n is 2-5, R₁ is one or two naturally occurring amino acids, optionally alpha-amino acetylated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₂ is 1 or 2 naturally occurring amino acids, optionally alpha-carboxyl esterified or amidated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms and X is R₃-L or D-His (R₄)-R₅ where R₃ is void or ω-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₄ is void or imidazole modification with alkyl-sulphydryl, hydroxyl, halogen and/or amino groups, and R₅ is void or carbonyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms. Preferably, the compound is carnosine.</p>		

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Method for the Treatment of the Complications and Pathology of Diabetes

The present invention relates to a method of treating the complications and pathology of diabetes.

5 The di-peptide carnosine, was discovered about 90 years ago (Gulewitsch and Amiradzibi, 1900) as a heat-stable extract derived from meat; since these early origins, considerable data has accumulated on the distribution and metabolism of the di-peptide. Carnosine
10 (β -alanyl-L-histidine) and its related compounds such as anserine (β -alanyl-L-methyl-L-histidine) and homocarnosine (γ -amino-butyryl-L-histidine) are present in millimolar concentrations in numerous mammalian tissues, including skeletal muscle (2-20mM) and brain
15 (0.3-5mM). Although no unified hypothesis exists to account for physiological function of this group of di-peptides, their antioxidant properties, ability to protect DNA from radiation damage, ability to chelate divalent cations, and remarkable buffer capacity at
20 physiological pH-values has led to the proposal that their primary function in vivo is to furnish protection to proteins, lipids and other macromolecules.

In addition to its role as free radical scavenger carnosine has been claimed to act as an "immunoregulator"
25 (Nagai, Patent: GB 2143732A) with useful properties in the treatment of certain cancers (Nagai, Patent DE 3424781 A1). Carnosine has also been suggested to be useful in the treatment of lipid peroxide induced cataracts (Babizhayev, 1989). There is also evidence that carnosine
30 can accelerate the process of wound healing.

Non-enzymatic Glycosylation

Free-radical damage is not the only process to affect the structure of proteins and nucleic acids.

Non-enzymatic glycosylation (glycation), the Maillard
35 reaction in food chemistry (Maillard, 1912, or browning

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reaction, involves reaction of amino groups with sugar aldehyde or keto groups to produce modified amino groups and eventually forming advanced- glycosylation-end -products (AGE-products). Although glycation is slow in vivo, it is of fundamental importance in ageing and in pathological conditions where sugar levels are elevated, e.g. diabetes.

It is possible to demonstrate glycation of proteins in the test tube. Several studies have shown that most proteins and DNA are potential targets for non-enzymic glycosylation in which sugars become attached to amino groups in the molecule via a Schiff's base. Subsequently a rearrangement occurs to give the coloured product (called the Amadori product). Further slow and uncharacterised reactions of the Amadori products occur.

Analysis of the preferred glycation sites in proteins shows the epsilon amino groups of lysine residues are primary targets, particularly when in proximity to histidine residues (Shilton & Walton, 1991). In a search for stable peptides with long half-lives in vivo we found that the amino acid sequence of carnosine is similar to Lys-His, thus having the potential to react with sugars and react as scavenger for aldehyde groups. In addition carnosine is virtually non-toxic; well documented toxicity studies have indicated that the material can be administered to mammals to a level of 5-10 g/kg body weight and therefore no toxic side effects are expected over long-term treatment.

So far only one other compound has been shown to slow down glycation by reacting with sugars and blocking the Amadori re-arrangement. Aminoguanidine can reduce both in vitro and in vivo glucose-derived advanced glycation end products. Unfortunately aminoguanidine, a nucleophilic hydrazine compound, is nonphysiological and is of unknown long-term toxicity.

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Diabetes

Diabetes is a metabolic disorder caused by an acute or chronic deficiency of insulin. It is diagnosed by an increased blood glucose level. The acute condition is characterised by a reduced glucose uptake of the insulin-dependent tissues. The body counteracts the resulting energy deficiency by increasing lipolysis and reducing glycogen synthesis. When the diabetic condition is severe, calories are lost from two major sources; glucose is lost in the urine, and body protein is also depleted. This is because insufficiency of insulin enhances gluconeogenesis from amino acids derived from muscle. The acute disorder can be controlled by insulin injections but since the control can never be perfect, the long-term fate of a diabetic is dependent on complications occurring later in life in the eye (cataractogenesis and retinopathy), kidney (nephropathy), neurons (neuropathy) and blood vessels (angiopathy and arteriosclerosis). It is well established that coronary heart disease is one of the most common causes of deaths in diabetics and non-diabetics alike.

Analyses of urine protein are usually requested in diabetic patients to rule out the presence of serious renal disease (nephropathy). A positive urine protein result may be a transient or insignificant laboratory finding, or it may be the initial indication of renal injury. The most serious proteinuria is associated with the nephrotic syndrome, hypertension, and progressive renal failure. In these conditions, the glomeruli become increasingly permeable to proteins by mechanisms that are poorly understood. The consequences are extremely serious, since they can progress rather rapidly to total renal failure and ultimate death. This form of proteinuria occurs as a secondary consequence to diseases like diabetes, amyloidosis and lupus erythematosus.

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As in other complications of diabetes, consideration of a potential role for glycation in the development of retinopathy must be taken into account. The retinal capillaries contain endothelial cells which line the capillary lumen and form a permeability (blood-retinal) barrier, and pericytes (mural cells), which are enveloped by basement membrane produced by the two cell types. Intramural pericytes are selectively lost early in the course of diabetic retinopathy, leaving a ghost-like pouch surrounding basement membrane. Breakdown of the blood-retinal barrier is another failure. Aldose reductase inhibitors are under investigation in treatment of experimental retinopathy in animals. Their mechanism of action is the prevention of the accumulation of sorbitol and resulting osmotic changes. However, the link with non-enzymatic glycosylation becomes obvious by the fact that Hammes et al (1991) have shown that treatment with aminoguanidine inhibits the development of experimental diabetic retinopathy. It is very likely that other potential inhibitors of glycation like carnosine should also have a positive effect.

Glycation and Atherosclerosis

Recent studies have suggested that AGE may have a role in the development of atherosclerosis. This is based on the finding that human monocytes have AGE specific receptors on their surface and respond when stimulated by releasing cytokines. Minor injury to the blood vessel wall may expose sub-endothelial AGE and promoting the infiltration of monocytes and initiating the development of atherosclerotic lesion. Circulating lipoproteins can also undergo glycation which is then taken up by endothelial cells at a faster rate than non-glycated lipoprotein. This is of importance in diabetes where an increased serum level of glycated lipoproteins has been reported. A compound with anti-glycation properties like

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carnosine should therefore have a positive effect on vascular diseases.

The reason for the diabetic complications are not fully understood as a continuous release of insulin after subcutaneous injections may not be adequate to respond to varying glucose concentrations necessary to avoid periodic hyperglycaemic conditions. Therefore, blood sugar levels in diabetics can be on average higher than in normal individuals resulting in an increased level of glycation. The best example are glycohaemoglobins which form non-enzymatically in red blood cells in amounts proportional to the cellular glucose levels. The higher percentage of glycated haemoglobin and serum albumin is used to monitor the degree of a diabetic's hyperglycaemia.

A controlled dietary intake of compounds which can counteract the long-term effects of high glucose levels in blood would be beneficial as an addition to a controlled diabetes therapies, such as insulin administration, sulfonylurea and biguanide treatment, on the use of amylin blockers. It is only the open chain form of reducing sugars like glucose, galactose, fructose, ribose and deoxyribose which participate in glycation. By scavenging this free aldehyde group and binding it in a non-toxic form we believe that it should be possible to decrease the damage caused by high sugar levels in vivo and in vitro. The compounds which are proposed for the treatment of the complications and pathology of diabetes could be peptides with one or more of the following characteristics:

- 1) they should be non-toxic even at relatively high doses;
- 2) they should be resistant to cleavage by non-specific proteases in the intestine and be taken up intact into the blood and organs, but should be cleared by kidneys, thereby following a similar tissue distribution to glucose in diabetes;

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- 3) the peptides should react rapidly with reducing sugars compared with amino groups on protein surfaces;
- 4) the resultant glycosylated peptides should not be mutagenic, in contrast to glycosylated amino acids,
- 5) if the peptide is cleaved by specific proteases in blood and tissue the resulting amino acids should be of nutritional value for diabetics, for example facilitating gluconeogenesis and counteracting a negative nitrogen balance.

10 Summary of the Invention

The present inventors believe that peptides having similar activity to that of canosine may be useful in the treatment of the complications and pathology of diabetes.

- Accordingly, in a first aspect the present invention consists in a method for the treatment of the complications and pathology of diabetes in a diabetic subject comprising administering to the subject a composition comprising a compound selected from the group consisting of (β -Ala-His)_n, (Lys-His)_n, a compound of the formula R₁-X-R₂, pharmaceutically acceptable salts thereof and combinations thereof; and a pharmaceutically acceptable carrier, in which n is 2-5, R₁ is one or two naturally occurring amino acids, optionally alpha-amino acetylated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₂ is 1 or 2 naturally occurring amino acids, optionally alpha-carboxyl esterified or amidated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms and X is R₃-L or D-His (R₄)-R₅ where R₃ is void or ω-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₄ is void or imidazole modification with alkyl-sulphydryl, hydroxyl, halogen and/or amino groups, and R₅ is void or carbonxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms.

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In a second aspect the present invention consists in the use of a compound selected from the group consisting of (β -Ala-His)_n, (Lys-His)_n, a compound of the formula R₁-X-R₂, pharmaceutically acceptable salts thereof and combinations thereof; in which n is 2-5, R₁ is one or two naturally occurring amino acids, optionally alpha-amino acetylated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₂ is 1 or 2 naturally occurring amino acids, optionally alpha-carboxyl esterified or amidated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms and X is R₃-L or D-His (R₄)-R₅ where R₃ is void or ω -aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₄ is void or imidazole modification with alkyl-sulphydryl, hydroxyl, halogen and/or amino groups, and R₅ is void or carbonxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, in the preparation of a medicament for the treatment of the complications and pathology of diabetes.

In a preferred embodiment of the present invention R₁ and R₂ are L- or D-lysine or L- or D-aspartic acid or L- or D-glutamic acid or homologues thereof. In a preferred form of the invention the compound is selected from the group consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine, and carcinine, it is presently most preferred that the compound is carnosine.

In a further preferred embodiment of the present invention the composition may include other compounds which have a beneficial effect in the treatment of the complications and pathology of diabetes, such as aminoguanidine.

Further, as a number of the subjects to be treated may also be on insulin sulfonylurea, biguanide or amylin blocker therapy the composition of the present invention

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may be co-administered with the insulin sulphonyurea, biguanide or amylin blockers therapy.

Further information regarding sulphonyurea acid biguanide therapy can be found in Beck-Nielsen
5 "Pharmacology of Diabetes", Eds. C.E. Mogensen and E. Standl, 1991, pp 75- 92, the disclosure of which is incorporated by reference.

Further information on the use of amylin blocker therapy in diabetes can be found in Westermark et al 1987
10 DNAS 84, 3881-3885, the disclosure of which is incorporated herein by reference.

One of the major drawbacks with insulin therapy is the continued need for injections. The present invention may provide an alternative in the oral administration of
15 carnosine with biguanides or sulphonyureas which may be more attractive to diabetics.

The composition of the present invention may be administered in any suitable manner such as injection, infusion, ingestion, inhalation, iontophoresis or topical
20 application. It is presently preferred, however, that the composition is administered orally.

In yet a further preferred embodiment the active compound is mixed with or linked to another molecule which molecule is such that the composition is improved in
25 regard to skin penetration, skin application, tissue absorption/adsorption, skin sensitisation and/or skin irritation. The molecule is preferably selected from the group consisting of sodium lauryl sulphate, lauryl ammonium oxide, ozone, decylmethylsulphoxide, lauryl
30 ethoxylate, octenol, dimethylsulphoxide, propyleneglycol, nitroglycerine, ethanol and combinations thereof.

It is also possible that the compound may be in the form of a prodrug. Further information on prodrug technology can be found in "A Text Book of Drug Design and
35 Development", edited by Povl Krogsgaard-Larsen and Hans

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Bundgaard, Chapter 5 "Design and Application of Prodrugs", H. Bundgaard. The disclosure of this reference is incorporated herein by cross-reference.

As stated above it is preferred that the composition of the present invention is administered orally. As will be understood by those skilled in the art numerous modifications can be made to the composition to improve the suitability of the composition for oral delivery. Further information on oral delivery can be found in "Peptide and Protein Drug Delivery" edited by Vincent H.L. Lee, Chapter 16 "Oral Route of Peptide and Protein Drug Delivery", V.H.L. Lee et al. The disclosure of this reference incorporated herein by cross-reference.

As stated above the composition may be administered by injection. Injectable preparations, for example, sterile injectable aqueous, or oleagenous suspensions may be formulated according to methods well known to those skilled in the art using suitable dispersion or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents which may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland, fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids, such as oleic acid find use in the preparation of injectables.

The total daily dose of the composition to be administered will depend on the host to be treated and the particular mode of administration. It will be understood that the specific dose level for any particular patient will depend on variety of factors including the activity of the specific compound employed, the age, bodyweight,

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general health, sex, diet, time of administration, route of administration, rate of excretion, and the severity of the particular side effects undergoing therapy. It is believed that the selection of the required dose level is well within the expertise of those skilled in this field.

It is believed that the dosage for carnosine would be in the range of 20mg to 2g/kg body weight/day and preferably in the range of 100mg to 200mg/kg bodyweight/day.

As stated above one of the complications associated with diabetes is cataracts. Accordingly, one particularly preferred mode of administration of the composition of the present invention is ophthalmic administration. In this situation the pharmaceutically acceptable carrier will be sterile aqueous or non-aqueous solutions, suspensions, emulsions and ointments. Examples of suitable pharmaceutically acceptable vehicles for ophthalmic administration are propylene glycol, and other pharmaceutically acceptable alcohols, sesame or peanut oil and other pharmaceutically acceptable oils, petroleum jelly, water soluble ophthalmically acceptable non-toxic polymers such as methyl cellulose, carboxymethyl cellulose salts, hydroxy ethyl cellulose, hydroxy propyl cellulose; acrylates such as polyacrylic acid salts, ethylacrylates, polyacrylamides, natural products such as gelatine, alginates, pectins, starch derivatives such as starch acetate, hydroxy ethyl starch ethers, hydroxy propyl starch as well as other synthetic derivatives such as polyvinyl alcohol, polyvinyl pyrrolidone, polyvinyl methylether, polyethylene oxide, carbopol and xanthan gum and mixtures of these polymers. Such compositions may also contain adjuvants such as buffering, preserving, wetting, emulsifying dispersing agents. Suitable preserving agents include antibacterial agents such as quaternary ammonium compounds, phenylmercuric salts, benzoyl alcohol, phenyl ethanol, and antioxidants such as sodium metabisulphide.

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Suitable buffering agents include borate, acetate, glyconate and phosphate buffers. The pharmaceutically ophthalmic compositions may also be in the form of a solid insert.

5 As will be clear from the foregoing discussion the complications and pathology of diabetes are treated by reducing or preventing non-enzymatic glycosylation. Accordingly, it could be expected that the method of the present invention would also be useful in the treatment of
10 other adverse complications and pathology of other disease states which are due to non-enzymatic glycosylation.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples
15 and figures in which:-

Figure 1 shows the reaction rate of L-carnosine with sugars. L-carnosine (60mM) was reacted with sugars (180mM) in 50mM na-phosphate buffer pH7 for five hours at 60° and the loss of the free amino group of carnosine assayed by
20 HPLC. SEM (± 1% of total carnosine in incubation mixture;

Figure 2 shows the effect of carnosine on atherosclerosis (■ cholesterol, □ cholesterol plus carnosine); and

25 Figure 3 shows the effect of carnosine on formation of cataracts in diabetic rats (■ control, ■ diabetic, □ diabetic plus carnosine).

Detailed Description of the Invention

Methods:

30 Reaction of Peptides and Amino Acid Derivatives with Sugars

Unless otherwise stated, reactions were carried out in phosphate buffered saline, PBS, (140mM-NaCl/ 10mM-sodium phosphate, pH 7.4) in sealed microcentrifugation vials in a 60°C waterbath. The reaction mixture contained 50mM
35 peptide and 500 mM sugar. At specified time points samples

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were taken, diluted 1:20 with water, and stored at -20°C prior to analysis by HPLC.

Detection of Amino Groups

- For the detection of free amino groups on peptides the
- 5 Waters AUTO. OPATM was used (Waters AUTO.TAGTM operation manual). In brief, peptides were reacted with o-phthalaldehyde and the fluorescent derivative separated by HPLC on a Radical-PAKTM C₁₈ column using a 10% (v/v) to 90% (v/v) methanol gradient over 15 min as solvent. A
- 10 Waters 470 fluorescence detector set at excitation 340nm/emission 440 nm was used.

Gelfiltration Chromatography of Glycated Proteins

HPLC Specifications:

- COLUMN Superose 6, Pharmacia
- 15 SAMPLE: 100 μ of Protein Sample (Approximately 100 μ g)
- ELUENT: 10mM phosphate buffer pH 7.38, 140 mM NaCl, 2mM KCl, 0.02% NaN₃, 0.05% Tween 20
- FLOW RATE: 0.5ml/min
- 20 DETECTION: 280nm.

Calibration was performed using Pharmacia high molecular weight (HMW) and low molecular weight (LMW) calibration kit.

25	CALIBRATOR COMPONENTS		MOLECULAR	RETENTION	
			WEIGHT	TIMES	
HMW	Blue Dextran		>2,000,000	15.49	n=5
	Thyroglobulin		669,000	25.84	n=6
	Ferritin		440,000	29.73	n=5
	Catalase		232,000	32.58	n=6
	Aldolase		158,000 Co-elute	32.58	n=6
30 LMW	Blue Dextran		>2,000,000	15.65	n=5
	Albumin		67,000	33.74	n=6
	Ovalbumin		43,000	35.57	n=5
	Chymotrypsin A		25,000	39.23	n=6
	Ribonuclease A		13,700 Co-elute	39.23	n=6

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Analysis of mutagenic potential of glycated compounds,
"Ames Test"

Preparation of glycated compounds was performed according to Kim et al. (1991). In brief, D-glucose (1M) and each of the following: L-carnosine, L-lysine, L-alanine (all 1M) were dissolved in distilled water, the pH adjusted to 7 and the mixtures heated at 100°C for 80 min. The solutions (50µl and 250µl) were evaluated against strain TA 100 of Salmonella typhimurium using the plate incorporation method (Maron & Ames, 1983) with or without metabolic activation by a standard rat liver microsomal (S-9) preparation. 2-AF and 2-AAF were used as positive controls for the experiments with metabolic stimulation, otherwise sodium azide was included as strain specific positive control.

The Effect of Carnosine on Atherosclerosis

Male New Zealand white cross rabbits fed a high cholesterol (2%) diet were randomised to control or carnosine treatment 2% and plasma was assayed for cholesterol and triglycerides and carnosine. All animals received 100g of food pellets/day and free access to water.

At the end of the 8 week treatment period, the rabbits were anaesthetised with pentobarbitone (325mg/kg) and the total aorta removed. The arch, thoracic and abdominal regions were isolated by cutting the aorta circumferentially 1.0cm proximal to the first pair of intercostal arteries and 0.3cm above the coeliac artery. The adventitia was carefully dissected away, and the artery longitudinally cut to expose the intimal surface. The aorta was fixed in 10% buffered formalin for 48 hours. The lipid plaques in these vessels were then stained using Sudan IV and mounted with an aqueous mounting medium (Kaiser's Glycerol Jelly). The lesioned areas were directly traced from the mounted sections using an image analyser (Eye Com 850 image processor).

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Computer-aided planimetry was used to macroscopically determine the percentage of the affected area. Representative segments of atherosclerotic plaque were removed for confirmation by light microscopy. Results are expressed as mean \pm SEM.

Formation of Cataracts in Diabetic Rats

Male Sprague-Dawley rats weighing 200-250 g and 6 weeks of age were randomised to the following three treatment groups: control, diabetic, and carnosine-treated diabetic rats. Diabetes was induced with streptozotocin STZ (60mg/kg body weight in citrate buffer, pH 4.5, i.v.), and all animals with plasma glucose levels >20 mM after 1 week were included in the study. Diabetic rats were randomised to receive either no therapy or 2% carnosine in drinking water.

Results

Example 1

Reaction of Carnosine with Sugar

The rate of reaction between aldehydes and amino groups in glycation is dependent solely on temperature and reactant concentration, thus allowing the use of non-physiological conditions in in vitro experiments to speed up the reaction without affecting the equilibrium. The first step in the Maillard reaction, the formation of a Schiff base between aldehyde and primary amino group, varies according to amount of linear chain form of the sugar. This is followed by an Amadori rearrangement and complicated secondary reactions, most of which are poorly understood.

Incubation of glucose, galactose and dihydroxyacetone (DHA) with carnosine produced brown solutions characteristic of glycation as originally described by Maillard (1912). The reaction of carnosine with the sugars was followed by disappearance of free amino group measured flurometrically after HPLC. Glucose,

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galactose and DHA differed in their reaction with carnosine (Fig. 1). Glucose was the least reactive and DHA the most, showing at least a fifteen-fold difference. For convenience we chose to employ the triose DHA in most subsequent studies.

Example 2

Prevention of dihydroxyacetone induced modification of bovine serum albumin by carnosine

Physiological concentrations of bovine serum albumin (50mg/ml in 50mM Na-phosphate buffer pH 7.0) were incubated with or without 250mM dihydroxyacetone in the presence and absence of 250mM L-carnosine at 23°C for 4 weeks. The experiment was performed under sterile conditions and the ionic strength was the same in all vials. The results are shown in table 1.

In this long-term experiment dihydroxyacetone had glycated albumin and, as a result of an Amadori rearrangement, subsequently induced the formation of a solid gel. When carnosine was present the contents of the vial remained fluid.

Table 1

<u>Incubation conditions</u>	<u>Resultant effects</u>
Albumin + phosphate buffer	colourless, fluid.
Albumin + dihydroxyacetone	brown, firm gel.
Albumin + dihydroxyacetone	dark brown, fluid.

The effects of carnosine on dihydroxyacetone-induced non-enzymic glycosylation of bovine serum albumin.

Example 3

Comparison of the reaction rate of carnosine and different amino acids with glucose.

Slow non-enzymic glycosylation of proteins and nucleic acids by glucose can be accelerated in vitro by raising the temperature from physiological values to 50°C. The main targets of glucose in vivo and in vitro are basic amino acids lysine and arginine (either free or

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following their incorporation into proteins). Table 2 shows a comparison of the reaction of carnosine and different amino acids with glucose. In order to demonstrate the specificity of the Maillard reaction for reducing sugars, glucose was substituted by sorbitol (a non-reducing sugar). 500 μ l of glucose or sorbitol (250mg/ml in 50mM Na-phosphate buffer pH 7.0) were incubated with different amino acids or carnosine (500mM) at 50°C for 18 h. The optical densities at 400nm of the resultant solutions were measured (Table 2). Carnosine formed by far the most Maillard reaction product, approximately 2-times or 8-times more than the fastest reacting amino acid, L-lysine or beta-alanine respectively. A small amount of Maillard reaction product is also apparent when carnosine reacts with sorbitol, probably due to autoxidation of sorbitol to a reducing sugar.

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Table 2

Optical density at 400nm of Maillard reaction products
between dipeptides or amino acids with glucose or sorbitol

	<u>Incubation conditions</u>	<u>OD 400nm</u>
5	glucose + PBS	0.175
	glucose + carnosine	8.455
	sorbitol + PBS	0.000
	sorbitol + carnosine	0.209
	carnosine + PBS	0.041
10	glucose + D, L-alanine	0.266
	sorbitol + D, L-alanine	0.008
	glucose + beta-alanine	1.240
	sorbitol + beta-alanine	0.010
	glucose + L-arginine	0.469
15	sorbitol + L-arginine	0.010
	glucose + L-lysine	4.170
	sorbitol + L-lysine	0.009
	glucose + imidazole	0.046
	sorbitol + imidazole	0.035

20 Example 4

Reaction of carnosine, related peptides and amino acids
with dhydroxyacetone

When the reaction rates of carnosine, related
peptides and amino acids with DHA were compared (Table 3),
25 carnosine reacted faster than lysine, which suggested that
the dipeptide could compete against other sources of amino
groups for glycation. However, in this assay lysine had
two amino groups contributing to its reactivity whereas in
proteins only the epsilon amino group is usually
30 available. To compare the glycation rate solely of the
epsilon amino group, N-alpha-carbobenzoxyl-lysine
(Z-lysine) which as a blocked alpha amino group, was
used. When DHA was added to an equimolar mixture of
carnosine of Z-lysine the dipeptide reacted about ten

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times faster than the blocked amino acid (Table 3). The relative reactivity was retained when glucose was employed as the glycating sugar, although the experiment took ten days to complete(not shown). Ac-Lys-NHMe, a molecule which closely resembles a lysine residue incorporated into proteins, also showed a slower reaction with DHA compared to carnosine. The peptide Ac-Lys-His-NH₂ resembles the preferential glycation site in proteins and showed the same reactivity as does carnosine. The peptide beta-alanyl-glycine was virtually unreactive with DHA, confirming the requirement for histidine at position two in a peptide for fast glycation (Shilton & Walton, 1991). While D-carnosine (beta-alanyl-D-histidine) reacted as fast as the naturally-occurring isomer, the higher homologue, homocarnosine (gamma-amino-butyryl-L-histidine), reacted slower. This indicates that a minor structural change to carnosine (the addition of a methylene group) reduces its reactivity. It also becomes evident that modification of lysine by various groups has a significant effect on the reaction rate. Whereas Ac-Lys-NH₂Me (blocked amino and carboxyl group) reacted faster, Z-lysine reacted slower than the free amino acid.

It was also found that the addition of free imidazole and succinyl histidine (alpha-amino group blocked) promoted the reactivity of carnosine with DHA as shown by an increase in the rate of disappearance of the dipeptide's amino group. This is in agreement with the suggestions that imidazole either catalyses the Amadori rearrangement or reacts with an intermediate form thereby changing the equilibrium of the reaction towards AGE-products (Shilton & Walton, 1991).

Table 3

Compound		% reacted
5	A Beta-Ala-L-His-OH	26
	Beta-Ala-D-His-OH	26
	Ac-Lys-His-NH ₂	25
	AC-Lys-NH ₂ Me	21
	H-Lys-OH	17
10	Gamma-aminobutyryl-His-OH	15
	Z-Lys-OH	3
	Beta-Ala-Gly-OH	2

15	B Beta-Ala-L-His-OH + succinyl-His	33
	Beta-Ala-L-His-OH + imidazole	43

Glycation of Peptides and Amino Analogues by DHA.

A and B: Compounds were reacted with DHA in PBS for 5 hours at 60⁰C and the loss of free amino group assayed by HPLC. Data are expressed as percent of amino groups reacted with DHA (SEM \pm 1% of total peptide or amino acid in incubation mixture). B only: Succinyl-His and imidazole were added at equimolar concentrations to beta-Ala-L-His-OH and the amino group of the latter assayed.

Example 5

Mutagenic properties of glycated amino acids and glycated carnosine

Glycated amino acids such as lysine and arginine have been reported to be mutagenic (Kim *et al.*, 1991) in an assay system first described by Maron & Ames (1983) "Ames Test". Other glycated amino acids like proline and

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cysteine did not exhibit mutagenicity. We have investigated the mutagenicity of L-carnosine and the glycated forms of L-carnosine, L-lysine and L-alanine (Table 4). All four solutions appear to inhibit the indicator strain to some extent, especially at the 250 μ l dose. Our data confirm earlier results by Kim et al., (1991) that glycated L-lysine is mutagenic and may therefore be carcinogenic. The activity is slightly enhanced by the rat liver S-9 metabolic activation system. Glycated L-alanine showed no mutagenicity in our experiments and only weak mutagenicity in the earlier work. Both, free carnosine and glycated carnosine are not mutagenic. This would be anticipated should carnosine play a significant role in the Maillard reaction in vivo.

The reason for the difference of the glycated forms of L-carnosine and L-lysine is not known.

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Table 4

<u>Revertants per Plate with TA 100</u>				
Compound	Dose (μ l)	Without S-9	With S-9	
<hr/>				
5	L-carnosine	250	158 \pm 11	149 \pm 13
		50	154 \pm 14	179 \pm 15
	L-carnosine	250	142 \pm 17	158 \pm 19
	glycated	50	159 \pm 7	167 \pm 10
10	L-lysine	250	277 \pm 21	244 \pm 13
	glycated	50	357 \pm 17	553 \pm 19
	L-alanine	250	145 \pm 6	146 \pm 9
	glycated	50	160 \pm 9	181 \pm 10
<hr/>				
15	negative control		161 \pm 6	188 \pm 10
	+ azide		>1000	N/A
	+ 2AF		N/A	250 \pm 33
	+ 2AAF		N/A	500
<hr/>				

20 Mutagenic potential of glycated compounds
Salmonella typhimurium TA 100 indicator strain
his⁻ to his⁺ reversion system. Data represent the
mean number of revertants per plate and their standard
deviation for the test solutions and controls with and
25 without metabolic stimulation by rat liver microsomal
(S-9) preparation.

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Example 6Comparison of Carnosine with Aminoguanidine as Inhibitors for Non-enzymatic glycosylation.

To compare the effect of both carnosine and aminoguanidine on glycation bovine serum albumin (BSA) and ovalbumin was incubated with a constant amount of DHA and varying concentrations of either anti-glycator at 60°C. At the start of the reaction and after seven hours aliquots were taken and the progress of the reaction analysed by gel filtration on a Superose 6 column. Crosslinking or fragmentation of protein became clearly visible as a change in retention time compared to the untreated protein used as control. Some compounds eluted after theoretical retention time for the smallest compound. They tend to interfere with the column resin even at high ionic strength and presence of a detergent (Tween 20). They are not necessary small compounds but rather highly charged and reactive. Table 5 summarises the data. Both compounds seem to react differently in this system: Carnosine reduced formation of high molecular weight compounds and was slightly more effective at low concentration compared to aminoguanidine. In all aminoguanidine samples uncharacterised reaction products are formed predominantly at high concentrations (described as low molecular weight form "LMW" because the retention time is longer then observed for all other compounds). Since the albumin monomer peak area is also reduced it is most likely that these are reaction products between ovalbumin, aminoguanidine and DHA. All three compounds showed no change in retention time or peak area when incubated separately under the same conditions for seven hours. LMW were also observed when ovalbumin was replaced by bovine serum albumin in the incubation mixture (not shown). The LMW forms were never present in the carnosine samples. A good measure for the effectiveness of an

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TABLE 5

OVALBUMIN			
Percent Area of Chromatogram			
	HMW	Monomer	LMW
5 t ₀ hours			
Carnosine samples			
[A] to [D]	0	100	0
[control]	0	100	0
Aminoguanidine samples			
[A] to [D]	0	100	0
[control]	0	100	0
after 7 hours			
Carnosine samples			
[A]	9	91	0
[B]	6	94	0
[C]	3	97	0
[D]	25	75	0
[control]	68	32	0
Aminoguanidine samples			
[A]	0	31	69
[B]	8	20	72
[C]	0	41	49
[D]	38	40	22
[control]	68	32	0
25 Legend:	ovalbumin was incubated with DHA for 7 hours in the presence of various concentrations of either carnosine or aminoguanidine. Reaction products were separated on a gelfiltration column (Superose 6) and peaks grouped according to their retention times: HMW, high molecular weight (15-30 min); albumin monomer (35 min); and late eluting compounds LMW, low molecular weight (> 40 min). Carnosine and aminoguanidine concentration [control] 0mM, [A] 600mM, [B] 300mn, [C] 100mM, [D] 50mM.		
30			

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anti-glycator is the amount of unmodified ovalbumin remaining after 7 hours of reaction. Here carnosine was more effective at all concentrations compared to aminoguanidine.

5 Example 7

The Effect of Carnosine on Atherosclerosis

Coronary heart disease is one of the most common causes of deaths in diabetics and non-diabetics alike. Glycation has been implicated in the development of
10 atherosclerotic plaques in addition to many diabetic complications including diabetic kidney and eye disease. Cholesterol-fed rabbits were used to examine the effect of carnosine on atherosclerotic plaques formation over a period of 8 weeks. Our studies have shown that inhibition
15 of glycation with carnosine can reduce but not prevent plaque formation. These results are shown in Figure 2.

The two tailed P values for the data were calculated using the Mann-Whitney two sample test: thoracic aorta = 0.0529; abdominal aorta = 0.5368; aortic arch = 0.6623,
20 all data carnosine (n=6) feeding versus diabetic control (n=5). For comparison aminoguanidine gave the following results in a similar experiment: thoracic aorta = 0.12; abdominal aorta = 0.044; aortic arch = 0.067, all data
aminoguanidine (n=11) feeding versus diabetic control
25 (n=12). More animals were used in this study giving a statistically better result. However, there are clear indications that both inhibitors of non-enzymatic glycosylation can reduce plaque formation.

The body weight of the animals reduced over the
30 8 week treatment period, however there was no difference between the control and carnosine treated group. No difference in weight of various organs was observed in control versus carnosine treatment.

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	Body Weight in kg	
	Week 0	Week 8
Control	3.27 \pm 0.09	2.75 \pm 0.17
Carnosine	3.33 \pm 0.09	2.68 \pm 0.12

5	Weight of organs (g) after 8 weeks treatment		
	Liver	Kidney	Heart
Control	135.94 \pm 5.06	16.20 \pm 0.67	7.92 \pm 0.53
Carnosine	124.40 \pm 6.36	17.53 \pm 0.69	6.12 \pm 0.27

10 Example 8The Effect of Carnosine on the Formation of Cataracts in Diabetic Rats

Cataract is an opacification of the ocular lens sufficient to impair vision. Diabetes has been associated with cataract for many years and many laboratory experiments support the view that diabetes is the cause of one form of cataract. Diabetes in animals can be induced by streptozotocin and opacity of the lens starts to develop by 20 days after injection but dense opacities appear only after about 100 days depending on age at injection.

In cataract adducts of sugars to proteins including lens proteins have been identified and quantified. In most tissues there is little accumulation of late Maillard products even in diabetes but proteins in the lens nucleus have time not only to accumulate early glycation products but also for them to change into yellow Maillard products. The initial attack of a sugar leads to a variety of chemical entities and induces structural changes to enzymes, membrane proteins and crystallins in the lens and therefore several pathways can lead to damage. A compound like carnosine with its ability to scavenge the reaction aldehyde group of sugars should

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- reduce the onset of cataract. We have tested this in the streptozotocin induced diabetic rat model. After 8 week on a carnosine diet the animals showed a higher clarity (less opacity) compared to a diabetic control group (Mann Whitney two sample test, two tailed p value = 0.2092; carnosine feeding versus diabetic control) (see Figure 3). Since this was measured at 56 days, the half way mark of the experiment, the trend indicates a reduction in cataract formation by carnosine feeding.
- 10 Cataract can not only be induced by reducing sugars in animal models. Babizhayev (1989) has shown that lipid peroxidation can be one initiatory cause of cataract development in animal models. Injection of a suspension of liposomes prepared from phospholipids containing lipid
- 15 peroxidation products induces the development of posterior subcapsular cataract. According to his finding such modelling of cataract is based solely on lipid peroxidation and can be inhibited by antioxidants like carnosine. The formation of Maillard reaction products
- 20 however, is an independent pathway and cannot be influenced by antioxidants.

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Example 9The Effect of Carnosine on Proteinuria, Glycated Haemoglobin and Blood Glucose Levels in Diabetic Rats

At week 8 no significant changes were observed for the
 5 following parameters:

Albuminuria

<u>normal</u>	<u>diabetic</u>	<u>diabetic + carnosine</u>
2.4x/±1.3	2.51x/±1.07	2.51x/±1.48

Percent Glycated Haemoglobin (HbA_{1c})

<u>normal</u>	<u>diabetic</u>	<u>diabetic + carnosine</u>
1.5 ± 0.1	4.83 ± 0.23	4.49 ± 0.14

Blood Glucose (mM mean + SEM)

<u>normal</u>	<u>diabetic</u>	<u>diabetic + carnosine</u>
10.0 ± 1.5	29.84 ± 4.88	22.63 ± 3.00

15 Changes in proteinuria and retinopathy can only be
 observed after about 30 weeks of diabetic condition. The
 compound aminoguanidine, usually used for the prevention
 of non-enzymatic glycosylation does not reduce the amount
 of glycated haemoglobin in diabetic models even after 30
 20 weeks of feeding. This study is presently continuing.

It will be appreciated by persons skilled in the art
 that numerous variations and/or modifications may be made
 to the invention as shown in the specific embodiments
 without departing from the spirit or scope of the
 25 invention as broadly described. The present embodiments
 are, therefore, to be considered in all respects as
 illustrative and not restrictive.

Literature:

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CLAIMS:-

1. A method for the treatment of the complications and pathology of diabetes in a diabetic subject comprising administering to the subject a composition comprising a
5 compound selected from the group consisting of
(β -Ala-His)_n, (Lys-His)_n, a compound of the formula
 R_1 -X- R_2 , pharmaceutically acceptable salts thereof and combinations thereof; and a pharmaceutically acceptable carrier, in which n is 2-5, R_1 is one or two naturally
10 occurring amino acids, optionally alpha-amino acetylated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R_2 is 1 or 2 naturally occurring amino acids, optionally alpha-carboxyl esterified or amidated with alkyl or aralkyl of 1 to 12 carbon atoms,
15 preferably 2 to 6 carbon atoms and X is R_3 -L or D-His (R_4)- R_5 where R_3 is void or
ω-aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R_4 is void or imidazole modification with alkyl-sulphydryl, hydroxyl, halogen and/or amino
20 groups, and R_5 is void or carbonxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms.
2. A method as claimed in claim 1 in which the compound is selected from the group consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine,
25 D-carnosine and carcinine.
3. A method as claimed in claim 2 in which the compound is carnosine.
4. A method as claimed in claim 1 in which R_1 and R_2 are L- or D-lysine or L- or D-aspartic acid or L- or
30 D-glutamic acid or homologues thereof.
5. A method as claimed in any one of claims 1 to 4 in which the composition further comprises aminoguanidine.
6. A method as claimed in any one of claims 1 to 5 in which the composition is co-administered with insulin
35 sulfonylureas, biguanides and/or amylin blockers.

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7. A method as claimed in any one of claims 1 to 6 in which the composition is administered by injection, infusion, ingestion, inhalation, ophthalmically, iontophoresis or topical application.
- 5 8. A method as claimed in any one of claims 1 to 7 in which the composition is administered orally or ophthalmically.
9. A method as claimed in any one of claims 1 to 7 in which the compound is mixed with or linked to another
- 10 molecule which molecule is such that the composition is improved in regard to skin penetration, skin application, tissue absorption/adsorption, skin sensitisation and/or skin irritation.
10. A method as claimed in claim 9 in which the molecule
- 15 is selected from the group consisting of sodium lauryl sulphate, lauryl ammonium oxide, ozone, decylmethylsulphoxide, lauryl ethoxylate, octenol, dimethylsulphoxide, propyleneglycol, nitroglycerine, ethanol and combinations thereof.
- 20 11. A method as claimed in any one of claims 1 to 10 in which the compound is in the form of a prodrug.
12. The use of a compound selected from the group consisting of (β -Ala-His)_n, (Lys-His)_n, a compound of the formula R₁-X-R₂, pharmaceutically acceptable
- 25 salts thereof and combinations thereof; in which n is 2-5, R₁ is one or two naturally occurring amino acids, optionally alpha-amino acetylated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₂ is 1 or 2 naturally occurring amino acids, optionally
- 30 alpha-carboxyl esterified or amidated with alkyl or aralkyl of 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms and X is R₃-L or D-His (R₄)-R₅ where R₃ is void or ω -aminoacyl with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, R₄ is void or imidazole
- 35 modification with alkyl-sulphydryl, hydroxyl, halogen

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and/or amino groups, and R_5 is void or carbonxyl (alkyl) amides with 1 to 12 carbon atoms, preferably 2 to 6 carbon atoms, in the preparation of a medicament for the treatment of the complications and pathology of diabetes.

- 5 13. The use as claimed in claim 12 in which the compound is selected from the group consisting of carnosine, anserine, ophidine, homocarnosine, homoanserine, D-carnosine and carcinine.
- 10 14. The use as claimed in claim 13 in which the compound is carnosine.
- 15 15. The use as claimed in claim 12 in which R_1 and R_2 are L- or D-lysine or L- or D-aspartic acid or L- or D-glutamic acid or homologues thereof.
16. The use as claimed in any one of claims 12 to 15 in which the medicament further comprises aminoguanidine.
17. The use as claimed in any one of claims 12 to 16 in which the medicament is co-administered with insulin sulfonylureas, biguanides and/or amylin blockers.
- 20 18. The use as claimed in any one of claims 12 to 17 in which the medicament is to be administered by injection, infusion, ingestion, inhalation, ophthalmically, iontophoresis or topical application.
- 25 19. The use as claimed in any one of claims 12 to 18 in which the medicament is to be administered orally or ophthalmically.
- 30 20. The use as claimed in any one of claims 12 to 19 in which the compound is mixed with or linked to another molecule which molecule is such that the composition is improved in regard to skin penetration, skin application, tissue absorption/adsorption, skin sensitisation and/or skin irritation.
- 35 21. The use as claimed in claim 20 in which the molecule is selected from the group consisting of sodium lauryl sulphate, lauryl ammonium oxide, ozone, decylmethylsulphoxide, lauryl ethoxylate, octenol,

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dimethylsulphoxide, propyleneglycol, nitroglycerine,
ethanol and combinations thereof.

22. The use as claimed in any one of claims 12 to 21 in
which the compound is in the form of a prodrug.

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REACTION OF CARNOSINE WITH SUGARS

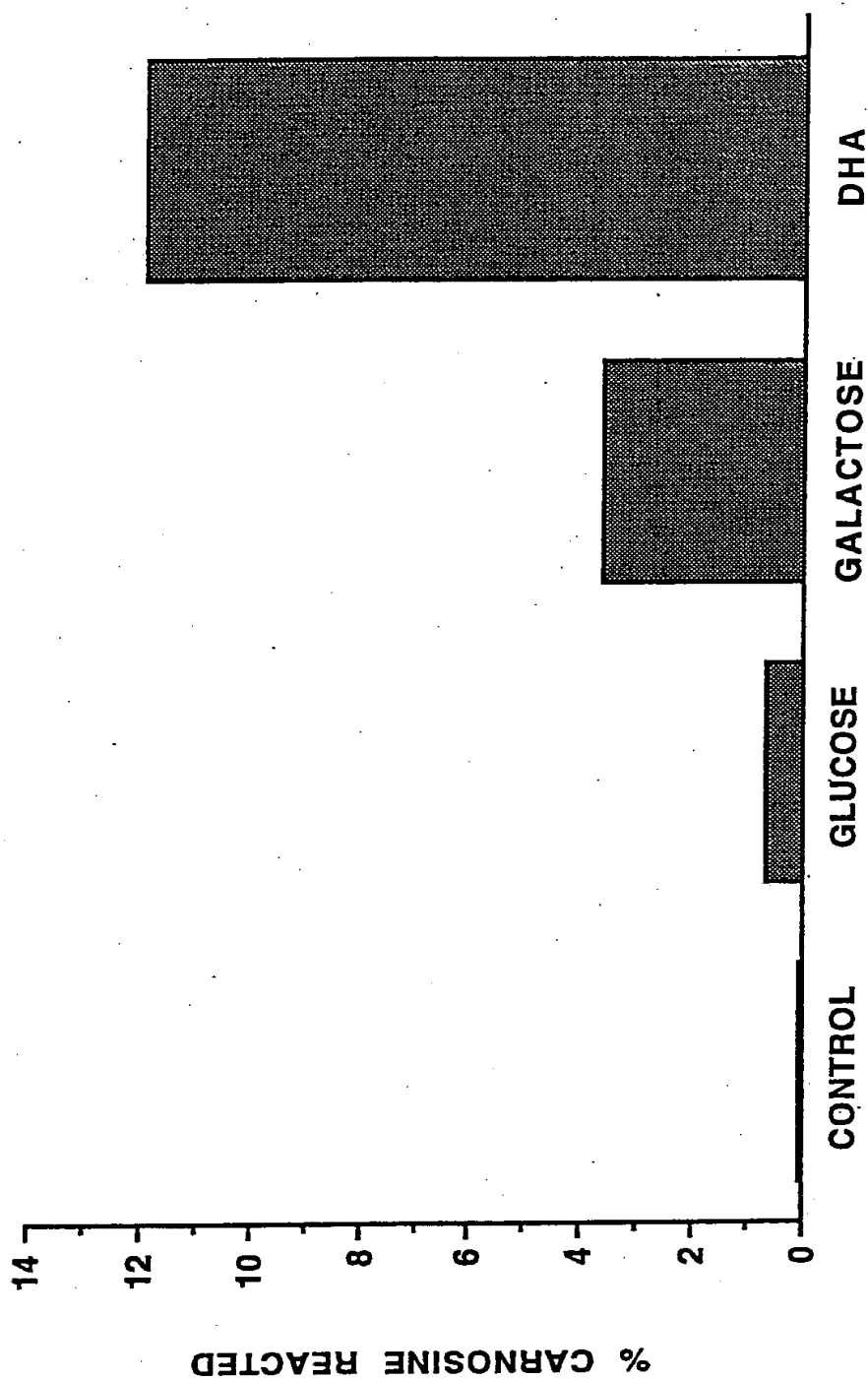


FIGURE 1

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ATHEROSCLEROSIS

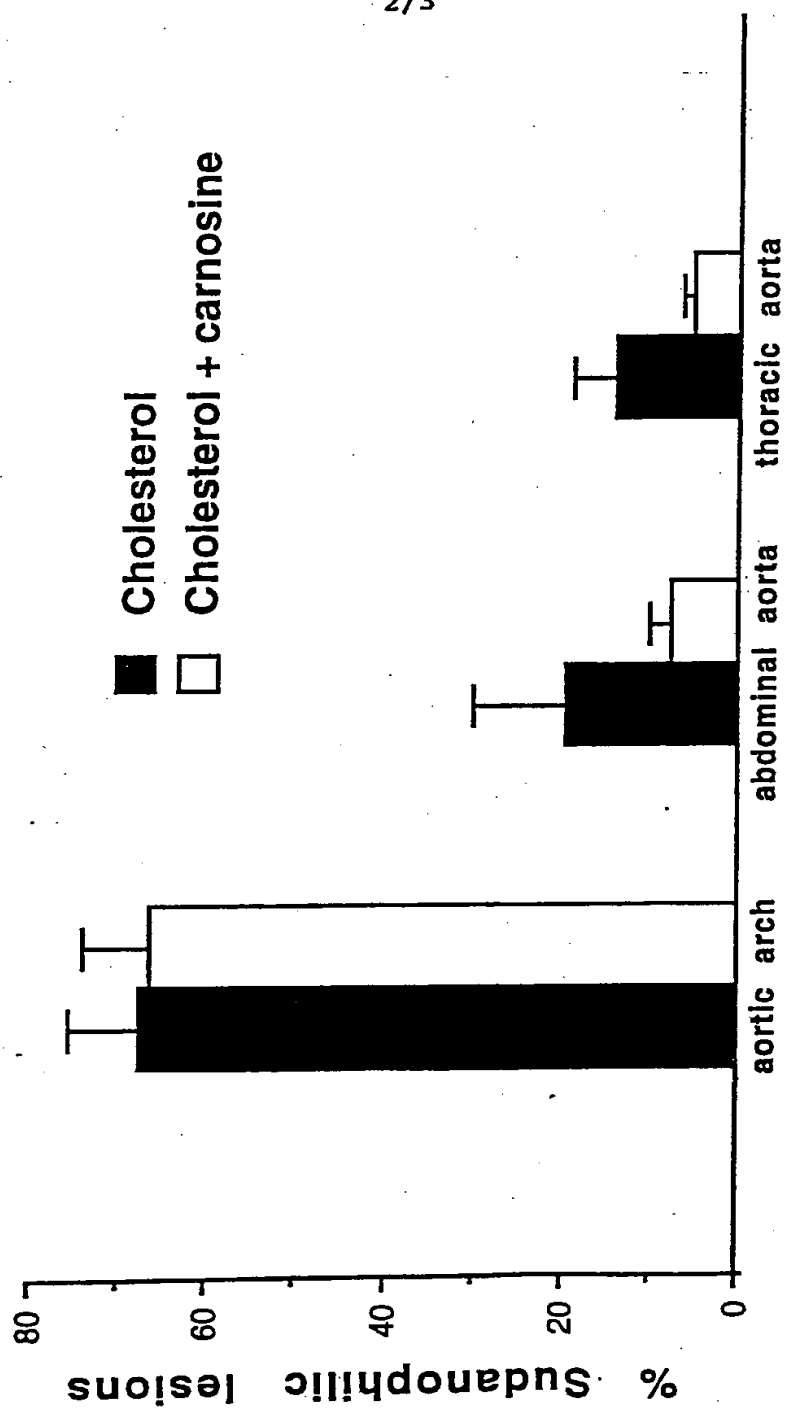


FIGURE 2.

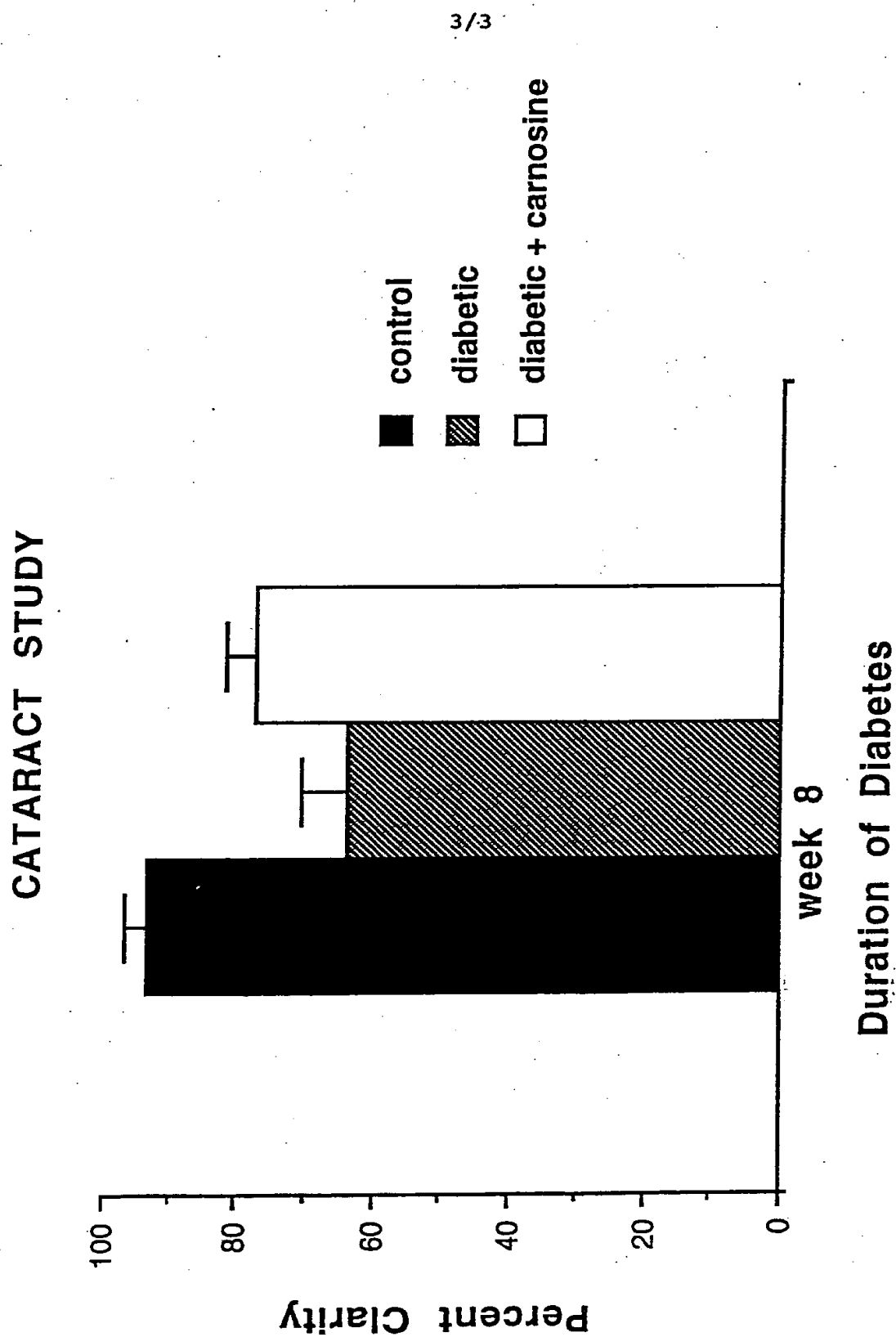



FIGURE 3.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00480

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ A61K 37/02 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC ⁵ A61K 37/02 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT: CHEM ABSTRACTS AND WPAT DATABASES USING THE KEYWORDS: CARNOSINE, HOMO CARNOSINE, ANSERINE, HOMO ANSERINE, OPHIDINE, CARCININE AND DIABETES				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.		
X	EP,A,313564 (ZERIA PHARMACEUTICAL CO., LTD. AND HAMARI CHEMICALS, LTD.), 3 May 1989 (03.05.89) page 5 lines 11-16, page 7 lines 2-8	12-15, 18-21		
X	EP,A,303380 (HAMARI YAKUHHIN KOGYO KABUSHIKI KAISHA) 15 February 1989 (15.02.89) page 2 lines 43-49, and the claims	12-15, 18-21		
X	US,A,4717716 (KINESHIRO NAGAI AND TAIKO SUDA), 5 January 1988 (05.01.88) column 8 lines 39-68, columns 9-10 lines 2-8, and the claims	12-15, 18-21		
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div> <input checked="" type="checkbox"/> See patent family annex. </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>			
Date of the actual completion of the international search 15 December 1992 (15.12.92)		Date of mailing of the international search report 24 Dec. 1992 (24.12.92)		
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (06) 2853929		Authorized officer  TAMARA NIZNIK Telephone No. (06) 2832260		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00480

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	US,A,4508728 (KINESHIRO NAGAI AND KINUKO NAGAI), 2 April 1985 (02.04.85) column 5 lines 20-68, column 6, columns 7-11	12-15, 18-21
X	WO,A,90/06102 (PEPTIDE TECHNOLOGY LIMITED), 14 June 1990 (14.06.90) page 3 lines 11-21	12-15, 18-21

International application No.
PCT/AU92/00480

Patent Document Cited in Search Report			Patent Family Member		
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EP	313654	JP	63014728	US	4927817
US	4717716	CH	666188	DE	3540632
		FR	2577138	GB	2170707
		NL	8600112	SE	8505121
EP	303380	JP	1042471	US	4981846
US	4508728				
<p>END OF ANNEX</p>					